Distribution of Ribosomal Cistrons in the Nuclei of Ehrlich Ascites Tumor and Hepatic Cells from Mice

Ken Higashi, Taeko Kuragano, Noriko Hanasaki, Tadao Matsuhisa, and Yukiya Sakamoto

Department of Biochemistry, Institute for Cancer Research, Osaka University Medical School, 3 Dojimahamadori, Fukushima, Osaka, 553 Japan

SUMMARY

Previously, we found that gene expressions for ribosomal RNA differ in tumorous and normal cells, so we compared the distributions of ribosomal cistrons in subfractions of nuclei from Ehrlich ascites tumor and mouse liver cells. The mean number of nucleoli per nucleus is mouse liver (diploid cells) is 3.7 while, in hyperdiploid and hypotetraploid strains of Ehrlich ascites tumors, the means are 1.8 and 3.6, respectively. However, in spite of the relatively few nucleoli per nucleus in tumor cells, the amount of DNA in the nucleoli is increased significantly (three- to fourfold). The ratio of annealing of radioactive ribosomal RNA with nucleolar DNA was similar in tumor cells and mouse liver cells, so the total amount of ribosomal genes in individual nucleoli of tumor cells must be greater than in mouse liver cells, and this increase may be enough to compensate for the reduction of nucleolar number in the nuclei of tumor cells. It is probable that fusion of nucleolar organizers (rather than amplification of ribosomal genes) occurs in the nucleoli of tumor cells, because no significant difference was found in the numbers of ribosomal cistrons in whole nuclear DNA of tumor and normal liver cells. Several ribosomal genes in extranucleolar regions are not expressed in vivo but were detected by hybridization in vitro.

INTRODUCTION

The number of nucleoli per individual nucleus varies in individual cells (5). However, in general, it seems that rapidly growing and less differentiated tissues contain fewer nucleoli than do tissues that are not growing. For instance, only a few large nucleoli are observed in tumors and fetal liver, while up to 6 small nucleoli are found in adult mouse liver (19).

The nucleolar 45S ribosomal precursor RNA in a number of transplanted tumors contains less adenylc and more cytidylic acid (12, 17, 20) than that of normal or regenerating rat liver (15). Furthermore, differences in base sequences of mature rRNA of tumor and rat liver cells have been reported from our laboratory (8, 9) and elsewhere (22–24). The present investigations were part of the studies on differences in gene expression for rRNA in tumor and normal tissues. From the results, it seems probable that the reduction in the number of nucleoli in tumor cells might be due to fusion of chromosomes containing nucleolar organizers, since both the DNA content and the total amount of rDNA are increased in tumor nucleoli without any significant change in the proportion of rDNA in whole nuclear DNA. However, it is not known whether the observed differences in nucleolar organization are characteristic of tumor cells or merely reflect the actively proliferating cells.

MATERIALS AND METHODS

Animals and 32P Labeling. Male ddY-F mice (4 to 6 weeks old) were used, unless otherwise specified. Ehrlich ascites tumor cells 2N/Os (with 44 chromosomes) and 4N/Os (with 77 chromosomes) were used. Tumor cells (approximately 106 cells/animal) were inoculated i.p. into mice, and ascites fluid containing the tumor cells was withdrawn from the peritoneal cavity 5 or 6 days later. According to the paper of Baserga (1), the growth of tumor cells is still exponential at this time.

To label rRNA, we gave the mice 2 i.p. injections of 5 mCi of 32P with a 20-hr interval between injections. The mice were sacrificed 24 to 26 hr after the 2nd injection.

Preparation of Nuclei and Ribosomes from Mouse Liver. Mouse livers were perfused with cold 0.9% NaCl solution and homogenized with 2.3 mM sucrose containing 3.3 mM CaCl2. Nuclei were obtained by the procedure of Chauveau et al. (6).

For ribosome preparation, mice were starved overnight and then were sacrificed. Their livers were perfused and homogenized with 0.25 mM sucrose containing 50 mM Tris-HCl, 25 mM KCl, 5 mM MgCl2 buffer, pH 7.6. Homogenates (25%, w/v) were centrifuged at 10,000 X g for 10 min. The upper two-thirds of the supernatant was removed by syringe and mixed with sodium deoxycholate at a final concentration of 1.3%. Ribosomes were collected through 2 discontinuous layers of sucrose, as described by Blobel and Potter (3).

Preparation of Nuclei and Ribosomes from Ehrlich Ascites Tumor Cells. Ehrlich ascites tumor cells were treated briefly with cold, hypotonic 10 mM Tris-HCl, 10 mM KCl, 1.5 mM MgCl2 buffer, pH 7.5, to remove contaminating red blood cells. Then they were resuspended in the same buffer and allowed to stand for 10 min at 0–2°C. Nonidet P-40 (Shell Chemical Co., New York, N.Y.) was added at a final concentration of 0.2%. Ribosomes were collected through 2 discontinuous layers of sucrose, as described by Blobel and Potter (3).

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1 This investigation was supported by a grant from the Japanese Ministry of Education.

2 The abbreviation used is: rDNA, ribosomal cistron.
Ribosomal Cistrons in Tumor Cell Nuclei

min, and the precipitate was suspended in 0.34 M sucrose containing 3.0 mM CaCl$_2$, layered over 0.88 M sucrose, and centrifuged at 2000 x g for 20 min. The resulting precipitate was used as the preparation of purified nuclei.

The initial supernatant obtained by centrifugation of the crude preparation of nuclei was used to obtain ribosomes, following the same procedure used to isolate ribosomes from mouse liver (3).

Purification of Ribosomes and Extraction of rRNA. Before extraction of rRNA, ribosomes were freed from mRNA by treatment with puromycin (2). The run-off monoribosomes were dissociated into subunits, fractionated on a 5 to 20% sucrose density gradient containing 0.5 M KCl and 5 mM MgCl$_2$ (Chart 1), and pooled. The RNA was extracted with 0.25% sodium dodecyl sulfate and phenol at room temperature (22°). The rRNA was purified on a gradient of 10 to 35% sucrose containing 0.1 M NaCl-1 mM EDTA and 10 mM acetate buffer, pH 5.1 (Chart 1). The regions of 28 S rRNA were essentially free from contamination with 28 S rRNA (Chart 1B), but 18 S rRNA from 40 S ribosomal subunits was always contaminated with a small amount of 18 S rRNA (Chart 1B), but 18 S rRNA from 40 S ribosomal subunits was essentially free from contamination with 28 S rRNA (Chart 1C). The regions of 28 S and 18 S rRNA were pooled, and the RNA was collected by precipitation with ethyl alcohol containing 2% potassium acetate. The specific activity of 28 S rRNA from Ehrlich tumor cells was 15,000 to 20,000 cpm/pg RNA and that of 28 S rRNA from mouse liver was 3,000 to 5,000 cpm/pg RNA.

Fractionation of Nuclei into Nucleoli and an Extranucleolar Fraction. Purified nuclei were treated sonically in a Umeda sonicator (20 kc) for 10 sec to obtain extranucleolar DNA. The preparation was then layered over 0.88 M sucrose and centrifuged at 2000 x g for 20 min (16). The upper layer (0.34 M sucrose layer) was used as the extranucleolar fraction. The precipitate was resuspended in 0.34 M sucrose and treated sonically until no unbroken nuclei were detectable under a phase-contrast microscope (which usually took 150 to 200 sec with nuclei of tumor cells and 90 to 120 sec with those of mouse liver). The resulting preparation was layered over 0.88 M sucrose and centrifuged at 2000 x g for 20 min. This step was repeated once to reduce contamination of the nucleolar preparation with extranucleolar chromatin.

Extraction of DNA. DNA was extracted from nuclei and nuclear subfractions by the procedure of Marmur (11), with the use of slight modifications described by Quagliarotti et al. (18).

Hybridization Procedure. Hybridization was carried out essentially by the procedure of Gillespie and Spiegelman (7). Before hybridization, rRNA was heated at 80° for 3 min and then was rapidly cooled (18) and passed through nitrocellulose filters. Hybridization was carried out for 18 hr in 2.5 ml of 0.90 M NaCl and 0.09 M sodium citrate with 0.1% sodium dodecyl sulfate and appropriate concentrations of RNA. About 45 μg of DNA were trapped on the nitrocellulose filters (Millipore Corp., Bedford, Mass.) when 50 μg of denatured DNA were passed through at a concentration of 10 μg/ml. Results were calculated from the quantities of radioactivity resistant to RNase remaining on the filters after the hybridization reaction. The value for the amount of DNA was divided by 2 on the assumption that only 1 strand of DNA was transcribed as a strand.

RESULTS

Number of Nucleoli per Nucleus. The number of nucleoli was determined by means of a hemocytometer with a phase-contrast microscope after the nucleoli were dispersed by brief sonic treatment in the presence of 1.5 mM CaCl$_2$. The amount of DNA was determined by the method of Burton (4).
preparations by phase-contrast microscopy. The mean number of nucleoli per nucleus in mouse liver was found to be 3.7, ranging from 1 to 7 nucleoli per nucleus (Chart 2). The mean number increased to 5 or 6 in the liver of 8-month-old mice (when the number of tetraploid cells increases) and was rather difficult to determine in older mice.

The mean numbers of nucleoli per nucleus of hyperdiploid and hypotetraploid strains of Ehrlich ascites tumor cells (see “Materials and Methods”) were found to be 1.8 and 3.6, respectively (Table 1). The distribution of nucleoli per nucleus is shown in Chart 2. The number of nucleoli per cell increases in ploidy of Ehrlich ascites tumor cells, as observed with mouse liver by Shea and Leblond (19). Tumor cells contained fewer nucleoli per nucleus than did normal cells.

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>DNA (pg/nucleolus)</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ehrlich ascites tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2N/0s</td>
<td>0.74 ± 0.11</td>
<td>4</td>
</tr>
<tr>
<td>4N/0s</td>
<td>0.61 ± 0.15</td>
<td>6</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>0.19 ± 0.04</td>
<td>5</td>
</tr>
</tbody>
</table>

*a Mean ± S.E.

DNA Content of Nucleoli. The amount of DNA in tumor cell nucleoli was 3- to 4-fold more than that in normal liver cells (Table 2) [as reported for a transplantable rat tumor (16)].

Contents of Ribosomal Cistrons in the Nuclei and Nuclear Subfractions from Ehrlich Ascites Tumor and Mouse Liver Cells. Chart 3 shows the results of a series of experiments in which various amounts of homologous 32P-labeled 28 S rRNA were annealed with constant amounts of DNA of the nucleolar and extranucleolar fractions of Ehrlich ascites tumor cells. The plateaus of the curves for both nucleolar and extranucleolar DNA’s were obtained with approximately 5 mg of 28 S rRNA of Ehrlich tumor cells. When the same amount (50 mg) of heterologous DNA from Micrococcus lysodeikticus was immobilized on the filters, it annealed with a negligible amount of rRNA (less than 0.0015%). Thus the amount of rDNA in extranucleolar DNA (0.021%) was significant, compared with the amount of nonspecific annealing of heterologous DNA.

Quantitative results are listed in Table 3. The percentages of RNA annealed with nuclear DNA were similar in tumor and mouse liver cells (about 0.042%). This means that there was no...
lower with Ehrlich tumor cells (Table 3), but the reason for mouse liver annealed with extranucleolar DNA was somewhat and mouse liver cells. The percentage of 28 S rRNA from per nucleus, and abnormal gene expression for rRNA. marked enlargement of nucleoli, the reduction in their number analyses of the partial nucleotide sequences of rRNA (8,9, 22, was 0.032%, as expected from the molecular weight of 18 S rRNA from Ehrlich tumor cells with nucleolar DNA was much higher (3- to 4-fold) in tumor cells than in the 2 types of cell (Table 2). Therefore, the total content of rDNA was much higher (3- to 4-fold) in tumor cells than in normal mouse liver cells. Similar distribution of rDNA was observed in the hyperdiploid (2N/0s) strain of Ehrlich ascites tumor cells (data not shown). The percentage of hybridization of 18 S rRNA from Ehrlich tumor cells with nucleolar DNA was 0.032%, as expected from the molecular weight of 18 S rRNA (data not shown).

No significant difference was observed in the extents of hybridization of 32 P-labeled 28 S rRNA in Ehrlich tumor cells and mouse liver cells. The percentage of 28 S rRNA from mouse liver annealed with extranucleolar DNA was somewhat lower with Ehrlich tumor cells (Table 3), but the reason for this is unknown.

**DISCUSSION**

Evidence of possible differences between the rRNA of tumors and normal tissues is accumulating, mainly from analyses of the partial nucleotide sequences of rRNA (8,9,22,24) and of competition hybridization (23). It is thought that there might be some relationship in tumors between the marked enlargement of nucleoli, the reduction in their number per nucleus, and abnormal gene expression for rRNA. Therefore, it seemed to be important to see the distribution of rDNA in tumor cells.

Surprisingly, there have been no previous reports on the content of rDNA in nucleolar DNA from either mouse liver or Ehrlich ascites tumor cells and this is, therefore, the 1st report of the amount of rDNA in the nucleoli of these tissues. The percentage of rDNA, which is complementary to rRNA, in nucleolar DNA was 0.075% in mouse tissues, while in rat tissues it was reported to be approximately 0.20% (18,21). Moreover, the degree of enrichment of rDNA with nucleolar DNA was less in mouse liver than in rat tissue (21). Much of the purified nucleoli of mouse liver consists of light satellite DNA, *i.e.*, one-fourth of the nucleolar DNA from mouse liver consists of these repeating sequences of DNA (13). There is no evidence of light satellite DNA in the nucleoli from rats, so the difference between the amount of rDNA in the nucleoli of rat and mouse tissue may be partially due to the presence of satellite DNA in mouse nucleoli.

The number of nucleoli per nucleus is consistently less in both hyperdiploid and hypotetraploid strains of Ehrlich ascites tumor cells than in mouse liver cells (Table 1). However, the content of DNA in the nucleoli of tumor cells is raised (Table 2), as would be expected from the enlarged nucleoli seen in tumor cells. It seems unlikely that this increase is due merely to the presence of S and G2 cells. The proportion of rDNA in nucleolar DNA is similar in tumor and normal liver cells, so the total amount of rDNA per nucleolus in Ehrlich ascites tumor cells increased in proportion with the increase in DNA content of the nucleolus. This increase appeared to be enough to compensate for the reduction in number of nucleoli in tumor cells. However, it is not known whether all of the rDNA in the tumor nucleolus are transcribed simultaneously. If only rDNA increased, the proportion of rDNA in the nucleolar DNA should increase. The DNA content increased in parallel with the amount of rDNA per nucleolus in tumor cells so, in these cells, fusion of chromosomes that contain nucleolar organizers occurs. It is uncertain whether this fusion is indispensable for the change in gene expression for rRNA or whether it is required only for the stimulation of biosynthesis of rRNA.

**Table 3**

<table>
<thead>
<tr>
<th>RNA hybridized (%)</th>
<th>28 S rRNA from tumor</th>
<th>28 S rRNA from liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ehrlich ascites tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear DNA</td>
<td>0.042 ± 0.008</td>
<td>0.069 ± 0.011</td>
</tr>
<tr>
<td>Nucleolar DNA</td>
<td>0.073 ± 0.006</td>
<td>0.011 ± 0.001</td>
</tr>
<tr>
<td>Extranucleolar DNA</td>
<td>0.023 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>Mouse liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear DNA</td>
<td>0.043 ± 0.010</td>
<td>0.065 ± 0.010</td>
</tr>
<tr>
<td>Nucleolar DNA</td>
<td>0.077 ± 0.021</td>
<td></td>
</tr>
<tr>
<td>Extranucleolar DNA</td>
<td>0.019 ± 0.005</td>
<td>0.024 ± 0.005</td>
</tr>
</tbody>
</table>

*Mean ± S.E.*
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Studies of regenerating mouse liver are in progress in our laboratory.

There are several rDNA's in the extranucleolar regions of the nuclei of mouse liver cells and Ehrlich tumor cells (Table 3), as reported by others for rat liver (10) and HeLa cells (14). They are probably latent genes in vivo that anneal with rRNA only after removal of chromosomal proteins. Comparative studies of the rDNA in both nucleolar and extranucleolar DNA's are needed to clarify the problem of the heterogeneity of rDNA.

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